are predominantly dispersive then it is a normal phase system. In practice, normal phase systems are used to separate mixtures of polar substances; they are largely ineffective in separating substances that are largely or exclusively dispersive. For reversed phase chromatography, the converse applies.

See also: **II/Chromatography: Liquid:** Column Technology; Theory of Liquid Chromatography.

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Mechanisms: Reversed Phases

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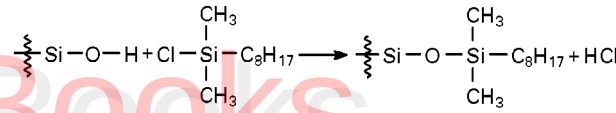
Introduction

In the early days of liquid chromatography (LC), the most commonly used stationary phase was silica gel, which was usually loaded with water and employed with a hydrocarbon mixture such as petroleum ether as the mobile phase. To change the selectivity of the distribution system, the hydrocarbon was sometimes adsorbed on the silica, and water or an aqueous alcohol mixture was used as the mobile phase. For obvious reasons, the latter was termed a reversed-phase system. Since that time, the reversed phase has been defined in a number of different ways: in this article, it is regarded as consisting of hydrocarbon moieties chemically bonded to a silica matrix. Reversed phases are considered to exhibit predominantly dispersive interactions with any solute or solvent with which they are in contact.

The first reversed phase was synthesized by Hálasz and Sebastian in 1969 by refluxing silica with a suitable alcohol (e.g. *n*-octanol) to form the silyl ester, the hydrocarbon chain being linked to the silica by carbon–oxygen–silicon bonds. This bonding proved to be labile, as the hydrocarbon moiety was easily removed by hydrolysis, but was sufficiently stable to allow the potential of such phases to be established. The next year Kirkland and DeStefano used chlorosilane reagents to attach the hydrocarbon chain to the silica by a silicon–oxygen–silicon bond which proved to be far more stable, at least between pH 4 and pH 8.



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Subsequently, other silyl reagents, such as the silyl esters, were also shown to react with silica. Some of these reagents are now commonly used in the production of reversed phases.

Brush Phases

There are three basic types of bonded phases which are produced by the use of the mono-, di- and trisubstituted silanes: brush phases, oligomeric phases and bulk phases. For example, the monochlorsilanes (e.g. octyldimethylchlorsilane) react with the hydroxyl groups on the silica surface to produce dimethyloctylsilyl chains attached to the silica. The alkyl chains are thought to stand out from the surface like the bristles of a brush, hence the term brush phase. After reaction, the material is usually treated with trimethylchlorsilane or hexamethyldisilazane to eliminate any remaining unreacted hydroxyl groups. This procedure is called capping the bonded phase.

The extent to which the silyl groups are reacted is still a subject of some debate. It is thought that the two methyl groups next to the silicon atom of the silyl reagent hinder reaction with adjacent hydroxyl groups on the silica gel surface. Consequently, a considerable amount of unreacted hydroxyl groups will

remain between the bonded moieties even after capping. It has also been suggested that one hydroxyl group may remain situated between each bonded chain. There is certainly evidence of some polar interactions with reversed phases which, if completely covered with hydrocarbon chains, should only exhibit dispersive interactions. However, all reversed phases are predominantly dispersive in character, and it would appear that if there are any hydroxyl groups still present on the surface, their influence on retention is relatively small compared with that of the bonded moieties. However, the residual hydroxyl groups, being strongly polar, can cause separation problems under certain circumstances and thus methods that reduce their effect have been developed and will be discussed later.

Oligomeric Phases

The di-substituted silanes such as methyloctyldichlorsilanes produce oligomeric bonded phases but this involves a more complicated procedure. The silica is first reacted with methyloctyl*dichlor*silane to link methyloctylchlorosilyl groups to the surface. The product is then treated with water, which hydrolyses the methyloctylchlorosilyl groups to methyloctyl*hydroxy*silyl groups with the elimination of hydrochloric acid. The hydroxy product so produced is then reacted with more methyloctyldichlorsilane, attaching another methyloctylchlorosilyl group to the previous groups. This process of alternately treating the product with the silane reagent and then water can be repeated until eight, 10 or more oligomers are linked to each other and attached to each sterically available hydroxyl group on the surface. It should be noted, however, that hydroxylation of the bonded moiety is not the only possible reaction that can take place with the water. There will be situations where it will be sterically possible for a water molecule to react with two adjacent chains and thus produce some cross-linking. However, a small amount of cross-linking would indeed strengthen the bonded system and could be advantageous.

The product is finally treated with trimethylchlorosilane, or some other capping reagent, to eliminate the last hydroxyl groups formed at the end of the oligomer. The oligomers are layered over the surface and this helps to exclude solutes from interaction with any residual surface hydroxyl groups, making the product extremely stable with almost no polar characteristics. However, due to the complexity of the synthesis (which, ideally, needs to be carried out in a fluidized bed for efficient reaction), oligomeric phases are expensive to manufacture and, consequently, are not generally available.

Bulk Phases

The trichlorosilanes (e.g. octyl*trichlor*silane) bond dichlorosilyl moieties to the surface and, in the presence of water, or if water is added, cross-linking occurs and a polymeric hydrocarbon stationary phase is produced. The same procedure can be used as that in the synthesis of oligomeric phases. The silica can be alternately treated with water and the trichlorosilane reagent. Layers of bonded phase are built up on the surface with extensive cross-linking, which produces a multilayer polymeric phase that has been termed a bulk phase. The bulk phases are almost as popular as the brush phases as they tend to have a higher carbon content (more organic material bonded to the surface) and thus provide a greater retention and selectivity. Bulk phases have about the same stability to aqueous solvents and pH as the brush phases.

The residual polarity of the bonded phase arising from the unreacted hydroxyl groups can cause peak tailing under certain circumstances. Consequently, it would be highly desirable to remove them or, at least reduce their effect on solute retention, particularly when present in brush phases. This was partly achieved by Kirkland who used reagents with larger base side chains (e.g. dipropyloctylchlorsilane as opposed to dimethyloctylchlorsilane) which linked a dipropyloctylsilyl group to the surface silicon atom. The larger propyl groups close to the silica surface tended to screen the adjacent hydroxyl groups sterically and thus significantly reduced their interactive availability. This, in turn, reduced their effect on retention. All three types of reversed phase are fairly stable, at least between pH 4 and pH 8 and at ambient temperature. The phase stability decreases as the temperature is raised: to some extent this can be overcome by introducing a phenyl group in the hydrocarbon chain.

Both brush and bulk reversed phases are commonly used in liquid chromatography, but the brush phase, although providing less retention for a given stationary phase loading, appears to be easier to manufacture in a reproducible form. Reversed phases are produced commercially with a range of chain lengths. Commonly available brush reversed-phase chain lengths range from a single carbon (using trimethylchlorsilane or ester as the reagent) to C_{30}.

In general, the retention of a solute increases linearly with the carbon content of the reversed phase, as shown in **Figure 1**. It is seen, however, that the brush phases and the bulk phases fall on different lines. The bulk phases both contain C_{18} chains but with different degrees of reaction; the ODS has a significant number of the hydroxyl groups unreacted. It appears that the hydrocarbon chains in the bulk

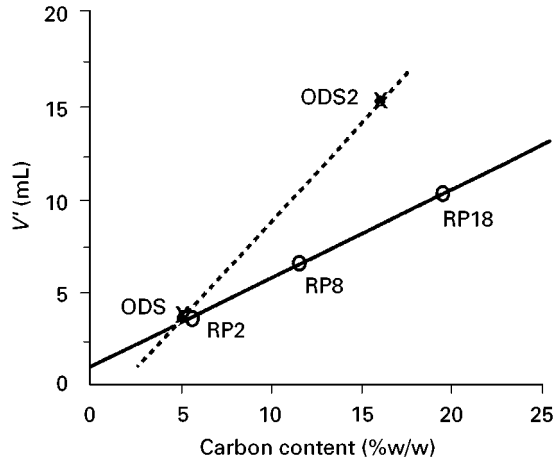

Figure 1 Graph of retention volume (V') against carbon (%w/w). ODS and ODS2, C_{18} bulk-reversed phases. RP2, RP8 and RP18, C_2, C_8 and C_{18} brush-reversed phases.

material are more available to the solute than in the brush material, indicating that in the bulk phases, the polymeric material might have a fairly open structure. The brush phases were fully reacted (although, as already discussed, some hydroxyl groups will still be present) and it is seen that retention increases linearly with the chain length.

Reversed phases with long chains and high retention properties, although apparently desirable due to their high selectivity, are not always appropriate. For example, the strong dispersive interactions exhibited by these phases can cause irreversible retention of proteins and other biopolymers, usually accompanied by denaturization. As a result, the C_2 and C_4 phases are very popular in the biotechnology field. In contrast, the separation of complex mixtures of stable compounds such as essential oils, fatty acids, pesticides, herbicides and the host of other mixtures produced and used in industry today are best achieved using the longer chain materials.

Particle Shape and Size

Originally, reversed phases were produced from irregularly shaped particles of silica which, after air jet grinding was introduced, were well rounded but not perfectly spherical in shape. The silica was cleaned by various washing procedures to remove heavy metals that were thought to cause peak tailing. This material, when converted to a reversed phase, packed well and provided columns of the expected efficiency (*c.* $L/1.6dp$, where L is the column length and dp the particle diameter). The introduction of spherical silica, which was claimed to pack more easily and was manufactured from silica of greater purity, has now largely replaced the irregular silica. In fact, the retentive properties and efficiencies derived from the spherical silica do not seem to be strikingly different from those produced from irregular silica, if packed properly.

Reversed phases are mostly produced in four particle sizes: 3, 5, 10 and 20 μm. The 3 μm material is usually provided in columns 3 or 5 cm long, giving about 5000 and 8000 theoretical plates respectively. These columns can be operated at 1–2 mL min^{-1} and thus provide fairly fast separations. The 5 μm packings are usually supplied in columns 5 and 10 cm long and the 10 μm particles in columns 10 and 20 cm long. A column 20 cm long packed with 10 μm particles can be expected to provide efficiencies of about 12 000 theoretical plates at a flow rate of 1 mL min^{-1}

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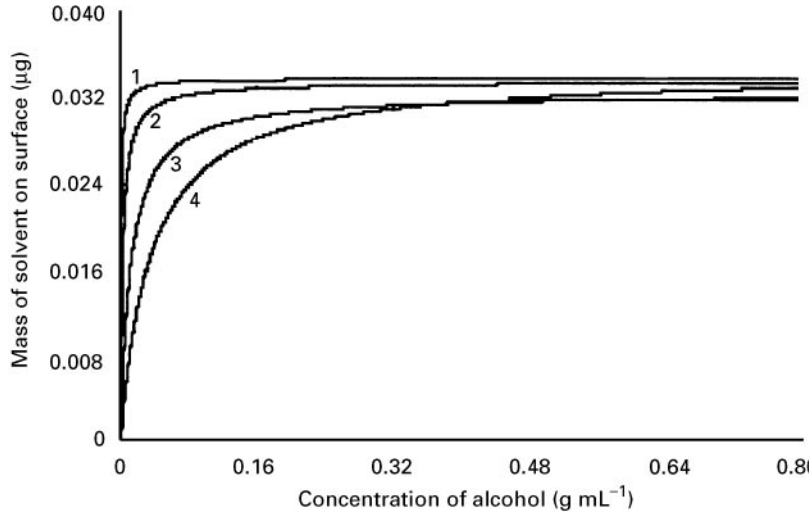

Figure 2 The adsorption isotherms for some aliphatic alcohols on a reversed phase. 1, Butanol; 2, propanol; 3, ethanol; 4, methanol. (Reproduced with permission from Scott, 1993.)

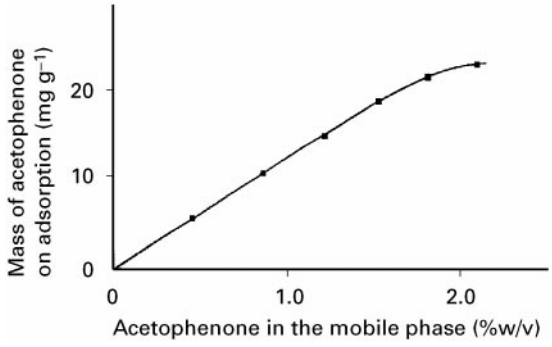

Figure 3 The adsorption isotherm of acetophenone between a reversed phase and an aqueous solvent mixture containing 40.4%w/v acetonitrile. (Reproduced with permission from Scott and Kucera, 1977.)

and is thus very useful in the separation of complex mixtures. The 20 μm packing is almost exclusively used for preparative columns.

Reversed-phase columns are slurry packed, usually employing a methanol–water mixture or ethanol as the packing solvent. There are many proprietary solvent mixtures used in packing reversed-phase columns and the technique has assumed an artificial cloak of mystery. The important aspect of packing is to use a wetting agent (there are many that can be used successfully) to ensure the particles do not clump together in the packing solvent but are completely dispersed. This can be checked under a low powered microscope. The packing, dispersed in the solvent and wetting agent (often by sonic vibration), is packed by suddenly applying about 8000 psi pressure to the particle dispersion contained in a high pressure reservoir, forcing the slurry through the column; the packing is retained by an appropriate terminal frit.

Interactions on the Surface of Bonded Phases

Solute interactions with the stationary phase in LC, differ from those in gas chromatography (GC) due to the preponderance of solvent that is concurrently interacting with the surface. In fact, in LC, the solute and the solvent interact in exactly the same manner with the stationary phase, but the concentration of the solvent is many orders of magnitude greater than that of the solute. Consequently, an equilibrium arises between the solvent and the stationary phase which is perturbed by the passing presence of the solute. It has been well established that solvents are adsorbed on the surface of a bonded phase as mono- or binary layers according to the Langmuir adsorption isotherm. The equation for the Langmuir adsorp-

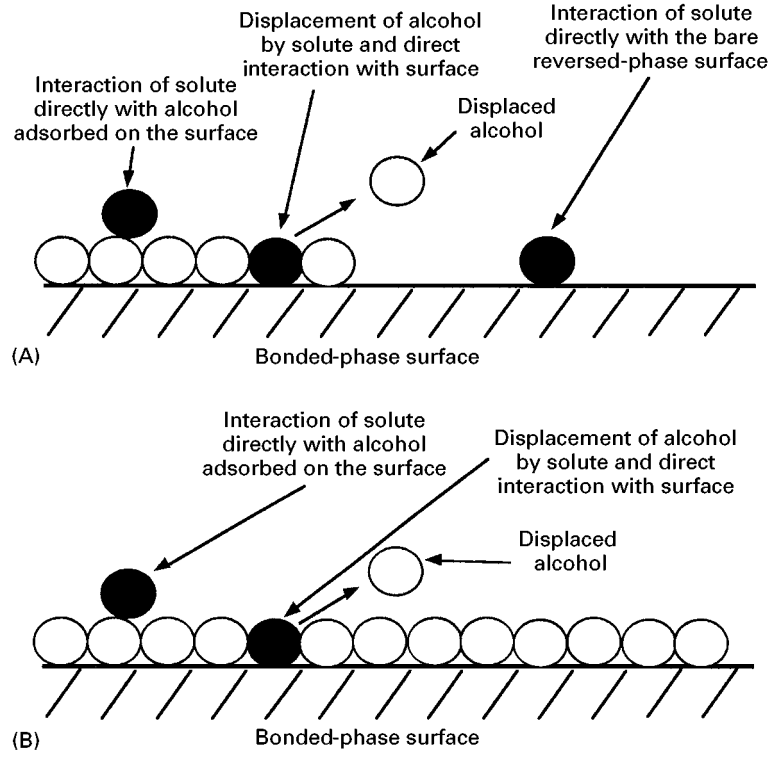
Displacement of alcohol by solute and direct interaction with surface
Interaction of solute directly with the bare reversed-phase surface
Interaction of solute directly with alcohol adsorbed on the surface
Displaced alcohol
(A)
Bonded-phase surface
Interaction of solute directly with alcohol adsorbed on the surface
Displacement of alcohol by solute and direct interaction with surface
Displaced alcohol
(B)
Bonded-phase surface

Figure 4 Alternative forms of solute interaction with the surface of a reversed phase. (A) Surface incompletely and (B) surface completely covered with solvent molecules. Open circles, alcohol; filled circles, solute.

tion isotherm of a monolayer is as follows:

$$\alpha = \frac{ac}{b + ac}$$

where α is the fraction of the surface covered by the solute, c is the concentration of the solute in the mobile phase, and a and b are constants.

At high concentrations α becomes unity and the surface is completely covered with the more strongly adsorbed solvent. An example of the monolayer adsorption isotherms of some aliphatic alcohols from their aqueous solutions is shown in **Figure 2**.

It is seen that the most polar alcohol, methanol, does not saturate the surface until the methanol concentration of the mobile phase is in excess of 30%. In contrast, the surface is completely covered with the more dispersive alcohol, butanol, when its concentration is less than 5%.

In reversed-phase chromatography, aqueous solutions of organic solvents such as methanol and acetonitrile are usually employed as the mobile phase. Consequently the solute molecules will also exhibit Langmuir-type adsorption isotherms when interacting with the surface of a phase. An example of the adsorption isotherm of acetophenone between a mobile phase containing 40.4% v/v acetonitrile (the concentration at which the reversed phase is completely wetted by the solvent) and the phase surface covered with adsorbed solvent is shown in **Figure 3**.

Only the initial approximately linear part of the isotherm is shown, indicating that the adsorption process is similar to that exhibited by the mobile-phase solvent. It is clear that the solute can interact with the reversed phase in a number of ways depending on the equilibrium conditions that exist between the solvent in the mobile phase and the surface. The alternative forms of interaction are depicted in **Figure 4**.

In the first case, where the concentration of the solvent is insufficient to cover the entire surface, the solute can interact directly with the uncovered surface, interact with the solvent molecules covering the surface or displace a solvent molecule and interact directly with the surface. All these alternatives will occur during the passage of the solute through the column. In the second case, where the concentration of the solvent is sufficient to ensure that the surface is covered with the solvent molecules, the solute can only interact with the solvent molecules covering the surface, or displace a solvent molecule and interact directly with the surface. The latter can only occur if the interaction energy between the solute and bonded moiety is greater than that between the solute and the adsorbed solvent.

It is seen that the interaction of the solute with the reversed phase can be quite complex and will depend not only on the nature of the solute itself, but on both the nature and concentration of the solvent in the mobile phase. It is clear that a ternary solvent system would be even more complex, and if the solvent composition is changed during development (as in gradient elution) the distribution system at any particular concentration, although possible to calculate from adsorption isotherm data, can be a very complicated procedure indeed.

Applications

Reversed phases are the most commonly used stationary phases in contemporary LC and are employed in over 65% of all analytical applications. They are largely used with water as a component of the mobile

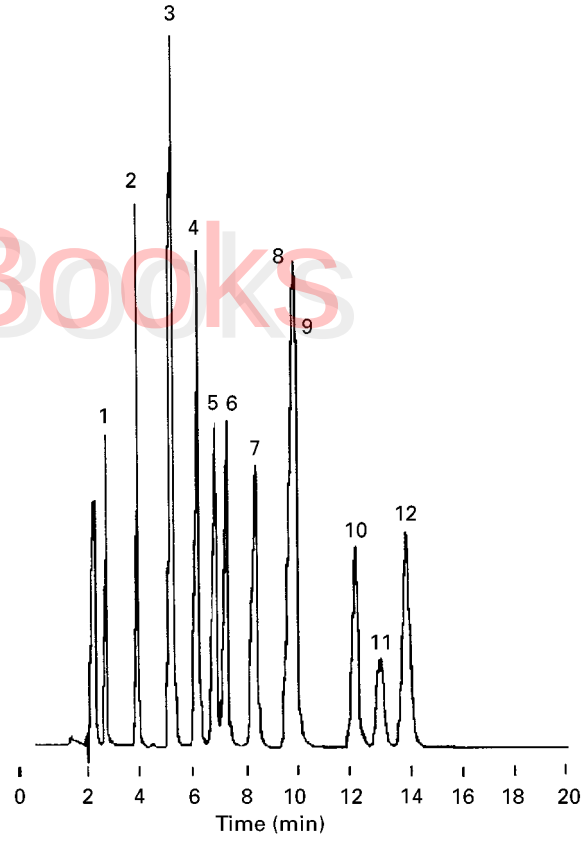

Figure 5 Separation of a sample of explosives. Column: 25 × 4.6 mm i.d. packed with C_{18} reversed phase. Particle diameter 5 μm. Solvent methanol–water, 50/50 at a flow rate of 1.5 mL min^{-1}. Sample volume 100 μL. 1, HMX (1 μg mL^{-1}); 2, RDX (1 μg mL^{-1}); 3, 1,3,5-trinitrobenzene (2 μg mL^{-1}); 4, 1,3-dinitrobenzene (10 μg mL^{-1}); 5, 2,4,6-trinitrotoluene (1 μg mL^{-1}); 6, tetryl (1 μg mL^{-1}); 7, nitrobenzene (1 μg mL^{-1}); 8, 2,6-dinitrotoluene (2 μg mL^{-1}); 9, 2,4-dinitrotoluene (1 μg mL^{-1}); 10, 2-nitrotoluene (0.1 μg mL^{-1}); 11, 4-nitrotoluene (0.5 μg mL^{-1}); 12, 3-nitrotoluene (0.1 μg mL^{-1}).

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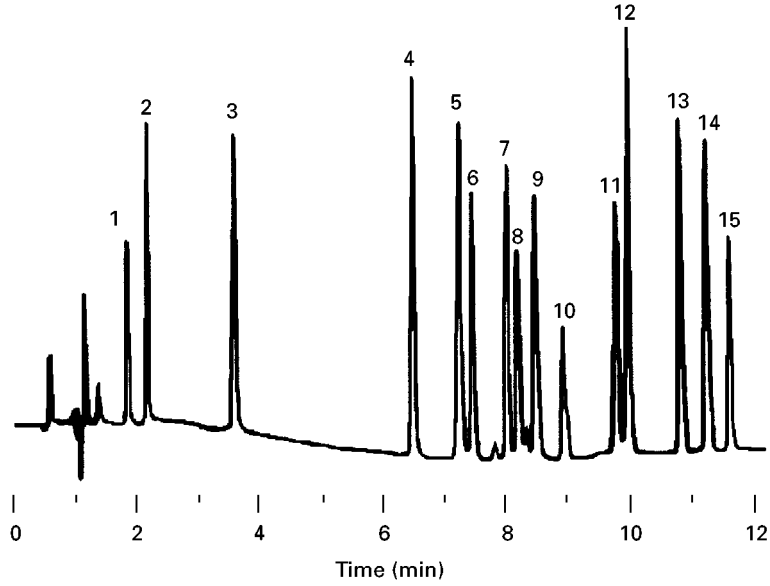
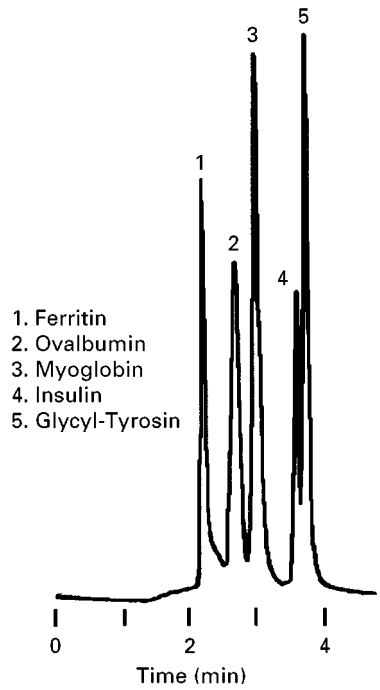


Figure 6 Separation of some urea pesticides on a C_8 reversed-phase column. Column LC 8 reversed phase, 15 cm long, 4.6 mm i.d., 5 μm particles used with a mobile-phase gradient of 18 : 82 acetonitrile–water to 65 : 35 to acetonitrile–water in 9 min. 1, Methomyl; 2, oxamyl; 3, fenuron; 4, manuron; 5, carbofuran; 6, propoxur; 7, carbaryl; 8, fluometuron; 9, duron, 10, propham; 11, siduron; 12, linuron; 13, chioprophan; 14, barban; 15, neburon.

phase, in binary, ternary and occasionally quaternary mixtures with aliphatic alcohols, nitriles and ethers, e.g. methanol, acetonitrile and tetrahydrofuran. They are also used with ion-pairing reagents in ion chromatography where the reagent is adsorbed as an interactive layer on the surface of the stationary phase. The mechanism of ion-pairing reagents is complex and will be discussed elsewhere.

Reversed phases are used in industrial product quality control, environmental testing, clinical analyses, biotechnological assays, forensic examination and many research programmes. Numerous examples of the use of reversed phases are included elsewhere in this book: some will be given here to illustrate the types of separation possible using predominantly dispersive interactions with the stationary phase. An example of an environmental or forensic type of application is given in **Figure 5**. The separation was carried out on an octadecyl bonded phase using a 50% v/v methanol–water mixture as the mobile phase. The selectivity of the dispersive stationary phase is clearly demonstrated, the more polar materials having strong polar interactions, with the strongly polar mobile phase being eluted first. In contrast, the relatively strong dispersive interactions of the monosubstituted toluenes with the reversed phase cause them to be eluted last. It is also seen that the explosive components are well resolved and detected quantitatively (mostly at the sub-microgram level) with no difficulty. Some samples can be separated by weaker dispersive interactions using reversed phases with shorter chains. An example of the use of a C_8 reversed phase for the separation of a number of carbamate and urea pesticides is shown in **Figure 6**. The separation is achieved using a gradient that starts with a highly polar binary aqueous mixture containing only 18% v/v acetonitrile to a moderately polar solvent mixture containing 65% v/v acetonitrile. It is seen that excellent separation is obtained in only 12 min.

As discussed earlier, the use of dispersive interactions to separate biologically labile compounds demands the use of short chain length reversed phases to prevent irreversible adsorption and/or solute denaturation. An alternative approach is to use one of the polar bonded phases in a manner where the dispersive interactions dominate but not sufficiently to cause strong adsorption. The amino or diol polar phases have been used in this manner to separate protein and protein-like materials and an example is given in **Figure 7**. It is seen that water containing no solvent is used as the mobile phase, which virtually completely negates the polar interactive capability of the polar stationary phase. The only significant interactions remaining are those with the dispersive moieties of the bonded material. This results in only weak interactions between the solutes and the stationary phase and the separation is attained without significant tailing (a symptom of strong adsorption) or denaturation.



An LC-Diol column, 25 cm long and 6.2 mm I.D. and a Mobile Phase of 0.1 M Potassium Phosphate Buffer at pH 6.8

Figure 7 The separation of some proteins on a polar phase used in the reversed phase mode.

Due to limited pressure available in liquid chromatographs, which is not constrained by pump design but rather by other parts of the chromatograph (in particular the sample valve), the reduction in particle diameter of a column packing is limited in practice. Small particles can only be used with shorter columns, which results in reduced separation times but not an increase in the attainable column efficiency. The shorter columns packed with very small particles also produce extremely small peak volumes that place serious constraints on the sample system and in particular the detector sensor cell. Although in the research laboratory, particles having diameters less than 1 μm may well be developed and their efficacy demonstrated, their general use in analytical laboratories will be very limited for some years to come. Due to the versatility and wide range of operating variables available, difficult separations are more easily achieved by using procedures other than by reducing the particle size to a level where practical difficulties become paramount.

Reversed phases are probably the most effective and popular stationary phases in use today. However, stationary phases employing bonded silica may well not be the best form of reversed stationary phase to use in the future. The great disadvantage to silica-based stationary phases is their instability in aqueous solvents, particularly at extreme pH. The future stationary phases that will be used in LC are more likely to be some form of macro-reticulated polymeric materials that have been developed and are already quite widely used. These polymeric particles can be prepared with significant surface area and porosity and are extremely inert and stable in aqueous solvent mixtures at the extremes of pH. They can also be prepared with a wide range of polarities linking different chemical groups to their surface and by using different polymer bases.

See also: **II/Chromatography: Liquid:** Column Technology. **III/Pharmaceuticals:** Basic Drugs: Liquid Chromatography. **Porous Polymers: Liquid Chromatography.**

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